STEM CELLS

Setting Standards for Human Embryonic Stem Cells

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Human embryonic stem cells (HESCs) are important both for their potential in regenerative medicine and as a window on early human embryology. A group of molecular biologists who study embryogenesis in human and animal systems gathered to review and assess molecular and cellular standards for HESCs—functional assays, and important future research avenues (*1*). This Perspective reports the proceedings of this workshop.

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Our goal was to initiate a scientific discussion that may help to establish standards for evaluating HESCs, in particular, to enable the comparison and classification of existing and future cell lines. This approach also should allow a direct comparison between HESCs and their counterparts in mice and other animals that are more amenable to experimentation. Molecular markers have enabled many new insights in the field of modern (nonhuman) embryology, and in a similar way HESCs may shed light on human embryology.

Basic Characteristics of HESCs

Embryonic stem cells are endowed with at least two remarkable properties. First, stem cells can proliferate in an undifferentiated but pluripotent state, and therefore can self-renew. Second, they have the ability to differentiate into many specialized cell types. HESCs are derived from the early human blastocyst (5 days postfertilization) from a region of the embryo called the inner cell mass (ICM). As with mouse embryonic stem cells (MESCs) (*2*–*5*), HESCs exhibit the following basic characteristics: (i) The cells are karyotypically normal, (ii) they survive and proliferate in vitro indefinitely under well-defined tissue culture conditions, (iii) most of the cells recover after freezing and thawing, and (iv) they differentiate into a variety of cell types in vitro and in vivo. These characteristics are well documented, but several important questions await resolution.

Reproducibility of HESC Derivation

Derivation of new HESCs in academic environments is limited by the moratorium on federal funding in a number of countries, including the United States. The pre-moratorium experience, combined with the experience of a few academic laboratories using private funds, as well as information available in the private sector and in countries where these experiments can be performed, suggests that scientists can derive HESCs with good success rates.

Effective Culture Conditions

Current protocols for dissociation of human embryos to generate stem cells are adapted from the mouse. Briefly, the ICM of a human blastocyst is removed by immunosurgery, dissociated in Ca^{2+}-Mg^{2+}–free medium, and plated over mouse embryonic fibroblasts or human feeder cells (*6*). The mouse cells are irradiated to suppress their proliferation. Current published culture conditions allow the maintenance of HESCs for many passages (up to 80) in the undifferentiated state (*7*, *8*).

Culture conditions enabling the successful generation of HESCs are similar to those for MESCs except regarding growth factor requirements. MESCs require the cytokine leukemia inhibitory factor (LIF) to maintain their undifferentiated pluripotent state, whereas HESCs are not responsive to LIF for the maintenance of pluripotency (*7*, *9*). An as yet unidentified signal in medium conditioned with mouse embryonic fibroblasts maintains HESCs, in a pluripotent state (*10*). In addition, HESCs, like other human cells in culture (but not MESCs) require fibroblast growth factor to grow (*7*). The longer cell cycle of HESCs in culture (*11*) may affect their responses in different ways from MESCs.

How Is HESC Self-Renewal Regulated?

LIF, which mediates its effect via the membrane protein gp130 and activation of the Stat3 signaling pathway, is sufficient to maintain the undifferentiated state of MESCs (*9*). In HESCs, LIF is not required to maintain the undifferentiated state, but whether or not the downstream signaling pathway (gp130, JAK/STAT) participates has not yet been studied. The extent to which signaling pathways are shared between HESCs and MESCs will determine the extent to which MESC experimental protocols must be adjusted for HESCs.

Are All HESC Lines the Same?

A related question is: How many states of "stemness" are there in the embryo? Progress in understanding these issues requires comparison of molecular markers of the undifferentiated pluripotent state as well as cellular characteristics and potency among different HESC lines. These studies are well advanced for MESCs but have not yet been initiated for HESCs (*9*). Thus, in-

CLASS I			CLASS II
GenBank	Unigene	Gene	
NM_002701	Hs.2860	Oct-3/4[7,8,10,12]	SSEA-3[7,12]
NM_003212	Hs 75561	TDGF1 (Cripto) [unpublished]	SSEA-4[7,8,10,12]
L07335	Hs.816	Sox2[12]	TRA-1-60[7,8,10,12]
NM_003240	Hs.25195	LeftyA [unpublished]	TRA-1-81[7,10,12]
AL558479	Hs 125359	Thy-1 cell surface antigen[12]	TRA-2-54[12]
BF510715	Hs.1755	FGF4[12]	GCTM-2[6]
NM_009556	Hs.335787	Rex1 (ZFP-42)[12]	

Molecular markers of HESCs. Listed are 13 molecular markers for identifying undifferentiated pluripotent HESCs. These markers are expressed (enriched) in undifferentiated HESCs and are turned off after differentiation. There are two groups: class I, cloned markers that are well defined and unambiguous, and class II, cell surface antigens detected by antibodies but requiring more extensive characterization.

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vestigators currently use molecular markers originally characterized in MESCs to test the state of an HESC line. These markers are defined as factors expressed consistently, and enriched, in embryonic stem cells. We propose two classes of markers (see the table): Class I markers are cloned, well defined, and unambiguous. Class II markers need more extensive characterization.

We note that not all mouse markers are expressed in human cells. For example, SSEA3 and SSEA4 are expressed in HESCs but not in MESCs, whereas SSEA1 is expressed in MESCs but not in HESCs (*12*). We anticipate that this list will grow rapidly as expression-profiling data from both MESCs and HESCs are further analyzed. The process could be expedited by making raw data from embryonic stem cell profiling available to the scientific community (*13*, *14*). Such state-specific expression fingerprints should help standardize our definitions of HESCs.

How Can HESCs Be Genetically Altered?

A number of reports have documented successful transfection and subsequent expression of genes in HESCs (*15*–*17*). Human retroviral vectors (such as lentiviruses) and biochemical strategies have shown promise for transfecting HESCs. If HESCs are going to be used for therapy, the possibility of random integration of transfected genes cloned into viral vectors should be addressed with care. Loss-of-function experiments by site-specific recombination (knockouts or knockins) routinely performed in MESCs have recently been reported in HESCs, and provide the opportunity to genetically alter HESCs (*18*).

How Robust Are HESCs?

We do not have a clear understanding of the molecular properties that underlie stem cell biology in any system, including mouse, and significant differences between MESCs and HESCs are already apparent. For instance, MESCs grow much more rapidly in culture than HESCs, the ligand involved in MESC self-renewal has been identified; MESCs can be derived from genetically manipulated backgrounds; and MESCs are much more amenable to a variety of in vivo assays free of ethical and sociopolitical issues. HESCs, on the other hand, are difficult to obtain and slow to grow; the molecular mechanisms underlying their self-renewal remain unknown. HESCs are also genetically difficult to manipulate and are very difficult to assay in vivo. Thus, basic questions about MESCs and HESCs should be addressed in parallel.

Additionally, because of the differences between MESCs and HESCs, the study of HESCs, rather than stem cells from model systems, is necessary if HESCs are to be developed for clinical applications.

How Well Do HESCs Differentiate?

In vitro, HESCs differentiate into a variety of cell types (but not all types) that derive from the three embryonic germ layers. Cultured HESCs form neurons and skin cells (indicating ectodermal differentiation) (*19*–*22*); blood, muscle, cartilage, endothelial cells, and cardiac cells (indicating mesodermal differentiation) (*23*–*25*); and pancreatic cells (indicating endodermal differentiation) (*26*)

Additionally, HESCs form embryoid bodies containing all three germ layers (*27*). Thus, HESCs exhibit an in vitro differentiation potential parallel to that of MESCs, but it is unclear whether this occurs during normal embryogenesis, or whether differentiation can be achieved through other pathways.

Developmental decisions follow a hierarchy in time. Before discrete organs and specialized cell types are made, the embryo (and, by inference, HESCs) generates three embryonic germ layers: ectoderm, mesoderm, and endoderm. In seeking the molecular pathways underlying organ formation, it is possible to identify activities that are necessary but not sufficient for the formation of a particular cell type. For example, a certain treatment might push HESCs to generate endoderm without giving rise to more specific endodermal derivatives, such as pancreas. Thus, it is important to follow differentiation in time to account for the intermediate steps a cell undergoes before it commits to its final fate. Understanding the normal progression of development should help us to design the sequence of treatments that can drive differentiation of embryonic stem cells. Although many markers of germ layers exist in model embryos, there is currently no comprehensive survey of these markers in human embryos.

An embryonic stem cell is defined as a cell that contributes to all embryonic germ layers and the germ line in vivo (*9*). The contribution to the germ layer of marked MESCs transplanted into mouse embryos at different stages of embryogenesis (most often at the blastocyst stage) is estimated by following the marked cells. A rigorous test of HESC potency in vivo is required.

For ethical reasons, the implantation of HESCs should not be performed with human embryos as hosts. The only published in vivo assay performed so far is the subcutaneous injection of HESCs into immunocompromised mice (such as NOD/SCID mice) (*7*, *8*). This assay revealed that HESCs behave like MESCs. The injected mice formed teratomas (embryonic-like tumors) that contain derivatives of the three embryonic germ layers. This in vivo assay is useful to assess HESC potency, but additional assays are needed to evaluate the contribution of HESCs to extraembryonic lineages and germ cells.

Analysis of HESC Differentiation

Improved in vitro and in vivo assays are essential for understanding the biology of HESC differentiation, a prerequisite for their use in regenerative medicine. Additional in vitro assays can be derived from those already in use for fish, frog, chick, and mouse embryonic tissues.

In vivo assays are more difficult to design. Drawing on lessons from model systems, there are two simple assays. First, marked HESCs could be transplanted into defined tissue environments in discrete regions of nonhuman adults or fetuses to test for their ability to be incorporated into these tissues. Similar experiments with mouse stem cells differentiated in vitro into motor neurons and transplanted back into a chick fetus have shown that these mouse neurons contribute to the chick spinal cord (*28*). Human fetal and adult neuronal and hematopoietic stem cells have been transplanted into mouse embryos, and their contribution to a variety of organs has been reported (*29*, *30*).

The first assay would test local contributions to specific fetal environments. In the second assay, marked HESCs could be transplanted at an earlier time point into nonhuman blastocysts to test for global incorporation into host tissues. HESCs transplanted into embryos of model systems, including mice and chickens, could be evaluated for their contributions to tissues and organs in these hosts. The assay should be performed transiently, that is, embryos would be removed from the host during different stages of gestation. We recognize that the second assay will require endorsement by the appropriate scientific institutional review board.

Infrastructure Requirements

Because the source of biological material (the human embryo) will always be limiting, the establishment of infrastructure is essential for effective progress. We suggest the creation of a HESC repository and registry, similar to the proposed Stem Cell Bank in the United Kingdom. The repository would collect all HESC lines (including those generated after 9 August 2001), test them for the criteria described above, and make them available to academic institutions. The quality controls and criteria for submission could be similar to those established for centers such as the American Type Culture Collection. This repository would take away the burden of obtaining the cell lines from

third parties and would assign the task to a centralized facility, which would take responsibility for providing the cells to interested laboratories in a timely fashion. This could provide a solution to the dilemma faced by U.S. researchers.

The registry would include a Web-based database available to university and private-sector researchers where all the data about HESCs and human embryos would be collected. The registry would collect and distribute information pertinent to a number of areas, including results of microarray analysis (raw data) or other high-throughput methodologies, growth and culture conditions of the cell lines, differentiation potential of the cell lines, and number of passages the cell lines can sustain.

Two aspects of this endeavor will require special attention. First, quality control of the deposited information (for example, raw microarray data) must be stringent, defined and imposed by a committee, which we suggest be composed of scientists with expertise in molecular embryology, high-throughput data analysis, and bioinformatics. Second, the maintenance and upgrade of the information will require a committed, long-term effort.

This document provides a starting point, which we anticipate will be refined and strengthened as our knowledge of HESCs and human embryology expands.

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ECOLOGY

Cryptic Herbivores of the Rainforest Canopy

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For decades, Edward O. Wilson has been telling the world that ants are the ecologically dominant animals of tropical rainforests. This assertion is a centerpiece of Wilson's message that ants are fascinating, that "little things" are important (*1*), and that tropical rainforests should be preserved. Since the 1970s, quantitative field studies have documented that ants constitute about 20 to 40% of the arthropod biomass in tropical rainforest canopies (*2*). Tobin (*3*, *4*) has questioned the ecological foundation that supports so many ants. Conventional wisdom holds that ants are primarily predators or scavengers, yet thermodynamic principles mandate that the greatest animal biomass in terrestrial communities must be the herbivores at the second level of the trophic pyramid. Thus, Tobin recognized a paradox and inferred that many ants in rainforest canopies must be herbivorous. A preliminary study provided provisional support for this fascinating hypothesis (*5*). Now, on page 969 of this issue, Davidson and colleagues (*6*) demonstrate that many rainforest ant species do, in fact, feed primarily or in part as herbivores. Cryptic herbivory may be the answer to Tobin's paradox.

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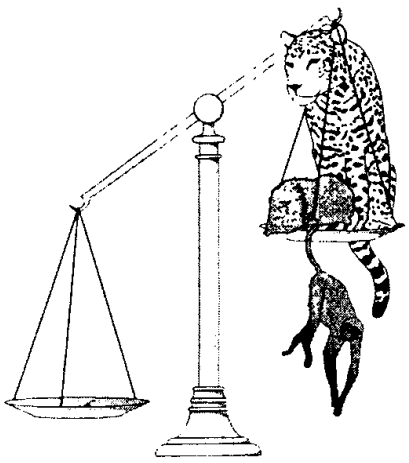
Protein is the principal currency of animal growth, with nitrogen as the essential element. The lighter of two stable isotopes of nitrogen is more readily lost in metabolic waste products, which causes the isotopic ratio of nitrogen in organisms to vary with ecological distance from the base of a food chain. Davidson and colleagues applied this analytical technique to ants, other insects, and plants from a Neotropical rainforest in Peru and a Paleotropical rainforest on the island of Borneo. Although the new data reveal that many rainforest ant species are predators or scavengers, many others have nitrogen isotope ratios in the same range as those of leaf-chewing insects, sap-feeding insects, and plants themselves. Overtly herbivorous rainforest ants feed on extrafloral nectar or the specialized food bodies of plants, or, in the case of Neotropical leaf cutter ants, on sap from the leaves that they cut. Unlike caterpillars and other leaf-chewing insects, no ants feed directly on leaf tissue. Ants that practice cryptic herbivory feed primarily on the liquid exudates of sap-feeding insects such as aphids, membracids, or scale insects (collectively called "trophobionts" when tended by ants). In addition, they may feed on pollen, fungal spores and hyphae, and leaf surface microflora (epiphylls). The ecological and evolutionary impact of ant herbivores, especially those that feed on the exudates of trophobionts, may be far-reaching.



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Tipping the scales of the rainforest carbon economy. The total biomass of ants in tropical rainforests exceeds that of mammals (*1*). Such abundance is hard to reconcile with the proposition that ants are primarily predators or scavengers and thus are two or more levels above plants in an ecological trophic pyramid. New research (*6*) shows that many rainforest canopy ants obtain nourishment primarily or substantially as herbivores. Herbivorous ants may be major players in the carbon economy of rainforests.

Close and sometimes complex ecological interactions between ants and plants drew attention primarily from myrmecologists (ant entomologists) until Janzen (*7*) elevated ant-plant symbioses (*8*) into the ecological mainstream. Most students of tropical ecology now confidently assert that ants provide a service to plants by acting as patrollers, keeping leaf-